Anwendungsbeispiel 2

Cytochrom P450 - Inhibition

Die Cytochrom P450 – Inhibition wurde entsprechend der Veröffentlichung von Crespi et al. (Anal. Biochem., 248, 188-190 (1997)) unter Verwendung von Baculovirus/ Insektenzellen-exprimierten, humanen Cytochrom P 450 Isoenzymen (1A2, 2C9, 2C19, 2D6, 3A4) durchgeführt.

Die Ergebnisse sind in der folgenden Tabelle dargestellt.

Hemmung der Cytochrom P450 Isoenzyme (IC50, µM)

Cytochrom P450 Isoenzym	1A2	2C9	2C19	2D6	3A4
Beispiel 2.54 der WO 00/27819	5,2	0,2	0,05	> 30	3,6
Beispiel 3.24	>10	10	5,2	3,3	14

Aus dem Ergebnis ist deutlich die überlegene Wirkung der erfindungsgemäßen Verbindungen gegenüber den bekannten Verbindungen zu erkennen.

teinbau auf 50% des ungehemmten Einbaus nach Abzug des Leerwertes (EDTA gestoppte Reaktion) zu hemmen.

[0112] Die Ergebnisse der Kinase-Inhibition IC50 in µM sind in der nachfolgenden Tabelle dargestellt:

Beispiel Nr.	VEGFR II (KDR) [µM]
3.24	0,012

Anwendungsbeispiel 2

Cytochrom P450 – Inhibition

[0113] Die Cytochrom P450 – Inhibition wurde entsprechend der Veröffentlichung von Crespi et al. (Anal. Biochem., 248, 188-190 (1997)) unter Verwendung von Baculovirus/ Insektenzellen-exprimierten, humanen Cytochrom P 450 Isoenzymen (1A2, 2C9, 2C19, 2D6, 3A4) durchgeführt.

[0114] Die Ergebnisse sind in der folgenden Tabelle dargestellt.

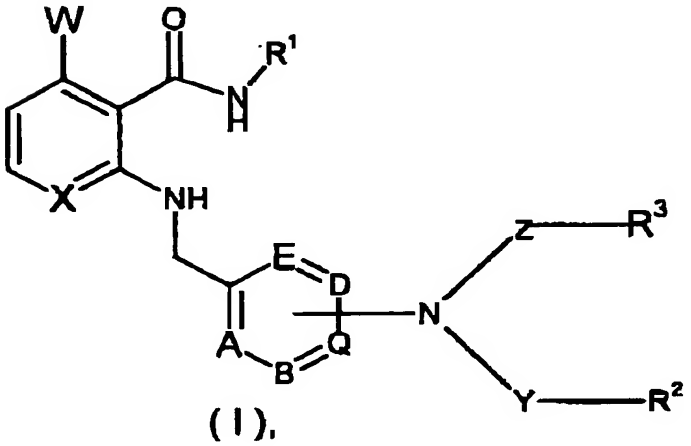
Hemmung der Cytochrom P450 Isoenzyme (IC50, µM)

Cytochrom P450 Isoenzym	1A2	2C9	2C19	2D6	3A4
Beispiel 2.54 der WO 00/27819	5,2	0,2	0,05	> 30	3,6
Beispiel 3.24	>10	10	5,2	3,3	14

[0115] Aus dem Ergebnis ist deutlich die überlegene Wirkung der erfindungsgemäßen Verbindungen gegenüber den bekannten Verbindungen zu erkennen.

Patentansprüche

1. Verbindungen der allgemeinen Formel I



(I),

in der
X für CH oder N steht,
W für Wasserstoff oder Fluor steht,
A, B, D,
E und Q jeweils unabhängig voneinander für ein Stickstoff- oder Kohlenstoff Atom stehen, wobei im Ring nur

gen digestion with commercial amyloglucosidase described above, a simple and reliable procedure for determination of both storage carbohydrates in whole yeast cells was developed, which can be readily applied to other microorganisms. Cells (4–10 mg dry wt) are collected by centrifugation (3 min at 5000*g* and at 0–4°C), carefully drained to remove the culture medium, resuspended in 0.25 ml of 0.25 M Na_2CO_3 using screw-top Eppendorf tubes, and incubated at 95°C for 4 h. The mixture is brought to pH 5.2 by addition of 0.15 ml of 1 M acetic acid and 0.6 ml of 0.2 M Na-acetate, pH 5.2. One-half of the suspension is incubated overnight with trehalase (0.05 U/ml) at 37°C, and the second half with *A. niger* amyloglucosidase preparation (1.2 U/ml) at 57°C, under constant agitation (for instance in a hybridization chamber). The suspensions are centrifuged for 3 min at 5000*g*, and glucose is determined on 20 μl (adequately diluted in water) of supernatant by addition of 200 μl of glucose oxidase mixture (Sigma, Cat. No. 510-A) and read at 420 nm in a ELISA reader apparatus. In our hands, this method appears much less constraining than the procedure of Schulze *et al.* (7) based on mechanical desintegration of cells in acetate buffer.

Using previous methods (2, 6, 7), the glycogen content can be overestimated due to the simultaneous presence of trehalose. The interference of trehalose in glycogen determination is not very significant during growth of yeast on glucose since trehalose accumulates later and at lower levels than glycogen (3, 9). However, it could be a serious problem when both carbohydrates accumulate together and in apparent equal amounts in culture media depleted of nitrogen, sulfur, or phosphorus sources (3, 7) or under stressful conditions (10).

As illustrated in Fig. 1A, glycogen was found to accumulate after transfer of the yeast culture from 25 to 42°C, when the assay of glycogen was carried out under the conditions prevailing in Becker's paper (6). However, when the incubation step was carried out at 57°C, there was no longer an increase in glycogen which then remained at the same level as at 25°C (Fig. 1B). Thus, it can be concluded that the rise of glycogen observed using a 37°C incubation of samples with amyloglucosidase was due in part to trehalose which otherwise accumulated at very high levels in yeast subjected to this temperature shock.

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Microtiter Plate Assays for Inhibition of Human, Drug-Metabolizing Cytochromes P450

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Cytochromes P450 are the principal enzymes for the oxidative metabolism of drugs and other xenobiotics. Among the xenobiotic-metabolizing cytochromes P450, five forms, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, appear to be most commonly responsible for the metabolism of drugs (1). Inhibition of cytochrome P450-mediated metabolism is often the mechanism for drug–drug interactions (2). The potential for enzyme inhibition is routinely assessed by performing *in vitro* inhibition studies using cDNA-expressed enzymes or human liver microsomes (3). These types of studies are becoming a routine part of the drug registration data package (4).

Combinatorial chemistry and high-throughput screening for pharmacologic activity can lead to the identification of relatively large numbers of compounds which have potential as human therapeutics (drug candidates). The availability of high-throughput assays for cytochrome P450 inhibition would facilitate the identification of those drug candidates which have a lower potential for drug–drug interactions (i.e., weak enzyme inhibitors). While microtiter plate-based fluorometric assays for CYP1A1 and CYP2B1 have been reported (5, 6), most assays for CYP2C9, CYP2C19, CYP2D6,

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TABLE 1

Kinetic Properties of the Substrate/Enzyme Pairs and Assay Parameters for Inhibition Analyses

Enzyme	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Substrate	CEC	CEC	CEC	CEC	BzRes
Apparent K_m (μM)	3.5	19	29	67	38
Apparent V_{max} (min^{-1})	3.4	0.004	0.016	0.017	0.3
Substrate concentration for IC_{80} (μM)	5	25	25	50	50
pmol enzyme per well	0.5[a]	20	5	5	5
Incubation time (min)	30	45	45	45	30

Note. Apparent K_m and V_{max} values were determined with the indicated enzyme concentration and incubation time using four to eight substrate concentrations and were calculated using nonlinear kinetics (GraFit 3.0). Actual ranges of substrate concentrations used were 0.4 to 50 μM, 3.1 to 50 μM, 0.8 to 50 μM, 6.25 to 50 μM, and 5 to 50 μM for CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 respectively.

[a] Control microsomes (prepared from insect cells infected with wild-type virus) were also added such that the final protein concentration was 0.05 mg/ml.

and CYP3A4 are relatively time consuming and labor intensive, usually requiring HPLC separation for metabolite quantitation. Therefore, there is a need for more efficient methods to assay these enzymes.

We have developed microtiter plate-based, fluorometric assays for the activities of five principal drug-metabolizing enzymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. Two direct fluorometric assays were used using 3-cyano-7-ethoxycoumarin [CEC[2] (7)] as a substrate for CYP1A2, CYP2C9, CYP2C19, and CYP2D6 and resorufin benzyl ether [BzRes (8)] as a substrate for CYP3A4. Baculovirus/insect cell-expressed enzymes of high catalytic activity permitted the use of these substrates which are slowly metabolized (per unit enzyme) for all enzymes except CYP1A2.

Assays were conducted in 96-well microtiter plates (Catalog No. 3598, Corning Costar, Cambridge, MA). The substrates, BzRes and CEC (Molecular Probes, Eugene, OR), were prepared as homogeneous suspensions in pH 7.4 to 7.5 buffer (potassium phosphate for all enzymes except for CYP2C9 which used Tris) by sonication (three bursts, Bransonic 250 sonifier, power level of 7). The substrate stock concentrations were twice the final concentration (final concentration chosen to be approximately the apparent K_m, Table 1). The 12 wells in a row were used for one inhibition curve. Wells 1 to 8 contained serial 1:3 dilutions of the inhibitors. Wells 9 and 10 contained no inhibitor and rows 11 and 12 were blanks for background fluorescence (stop solution was added before the enzyme). The final volume of substrate/inhibitor was 0.1 ml. Furafylline and sulfaphenazole were obtained from Ultrafine Chemicals (Manchester, UK). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Methanol was used to initially dissolve furafylline, sulfaphenazole, tranylcypromine, and ketoconazole. Final solvent concentration was less than 1%. Quinidine was dissolved in water. After substrate and inhibitor addition, the plates were prewarmed to 37°C. Incubations were initiated by the addition of 0.1 ml of prewarmed enzyme and cofactors (final incubation volume of 0.2 ml). The enzymes were commercially available, baculovirus/insect cell-expressed human cytochromes P450 (SUPERSOMES, GENTEST Corp., Woburn, MA). The amount of enzyme added per well is contained in Table 1. The final cofactor concentrations were 1.3 mM NADP, 3.3 mM glucose 6-phosphate, and 0.4 U/ml glucose-6-phosphate dehydrogenase (all from Sigma Chemical Co.). Final incubation volume was 0.2 ml. Incubations were carried out for 30 min (CYP1A2 and CYP3A4) or 45 min (CYP2C9, CYP2C19, and CYP2D6) and stopped by the addition of 0.1 ml of 60% acetonitrile, 40% 0.1 M Tris, pH 9. Metabolite formation was linear for these incubation times. Fluorescence per well was measured using a CytoFluor Model 2350 fluorescent plate reader (Millipore, Bedford, MA) controlled with an IBM-compatible 486DX2 computer. The CEC metabolite, 3-cyano-7-hydroxycoumarin, was measured using an excitation wavelength of 420 nm (50-nm bandwidth) and emission wavelength of 485 nm (20-nm bandwidth). The BzRes metabolite, resorufin, was measured using an excitation wavelength of 530 nm (25-nm bandwidth) and emission wavelength of 590 nm (35-nm bandwidth). Detection of the products of either assay was linear over the range used for these assays. Data were exported and analyzed using an Excel spreadsheet. The IC_{50} values were calculated by linear interpolation.

A series of enzyme-selective inhibitors (9) were examined with these assays (Fig. 1). The mechanism-based CYP1A2 inhibitor, furafylline (10), was a potent inhibitor of CYP1A2 with an IC_{50} of 670 ± 80 nM (mean ± SE, three determinations), with less than 50% inhibition of CYP2C9, CYP2C19, CYP2D6, or CYP3A4 being observed at concentrations up to 7500 nM (data not shown). The competitive CYP2C9 inhibitor, sulfaphenazole (11), was a potent inhibitor of CYP2C9 with an

[2] Abbreviations used: CEC, 3-cyano-7-ethoxycoumarin; BzRes, resorufin benzyl ether.

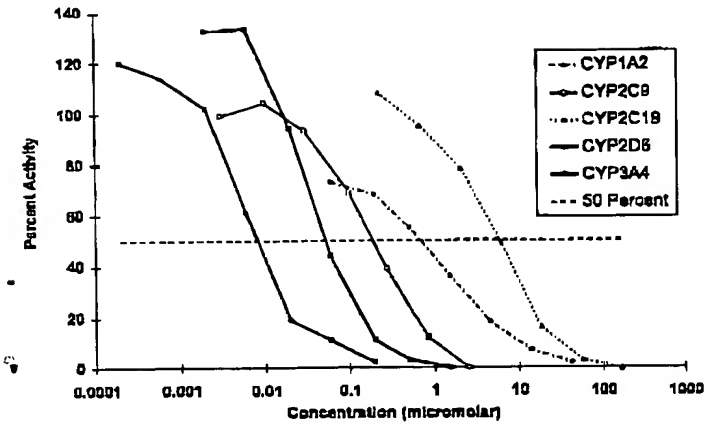

FIG. 1. Inhibition curves. Representative inhibition curves (wells 2 to 7) for furafylline/CYP1A2, sulfaphenazole/CYP2C9, tranylcypromine/CYP2C19, quinidine/CYP2D6, and ketoconazole/CYP3A4. The identity of the lines is provided in the figure inset.

IC_{50} of 230 ± 10 nM (mean ± SE, three determinations), with less than 50% inhibition of CYP1A2, CYP2C19, CYP2D6, or CYP3A4 being observed at concentrations up to 2000 nM (data not shown). The observed IC_{50} is in good agreement with the reported K_i for sulfaphenazole and human liver microsomal CYP2C9 [180 nM (11)]. Tranylcypromine inhibited CYP2C19 with an IC_{50} of 5800 ± 200 nM (mean ± SE, three determinations), with less than 50% inhibition of CYP1A2, CYP2D6, or CYP3A4 being observed at concentrations up to 32,000 nM. CYP2C9 was inhibited almost as effectively as CYP2C19 (data not shown). Limited enzyme specificity for this compound has been noted by others (9). Quinidine was a potent inhibitor of CYP2D6 with an IC_{50} of 8.9 ± 1.6 nM (mean ± SE, three determinations), with less than 50% inhibition of CYP1A2, CYP2C9, CYP2C19, or CYP3A4 being observed at concentrations up to 250 nM (data not shown). The observed IC_{50} is in agreement with the reported K_i for quinidine and human liver microsomal CYP2D6 [27 nM (12)]. Ketoconazole was a potent inhibitor of CYP3A4 with an IC_{50} of 83 ± 25 nM (mean ± SE, three determinations), with less than 50% inhibition of CYP1A2, CYP2C9, CYP2C19, or CYP2D6 being observed at concentrations up to 1250 nM (data not shown). The observed IC_{50} is in agreement with the reported IC_{50} for ketoconazole and human liver microsomal CYP3A4 [200 nM (13)].

Our observed IC_{50} values are in good agreement with those reported for the same enzyme/inhibitor pairs reported by others. Moreover, the form specificity of inhibition is maintained with this system [i.e., quinidine, sulfaphenazole, furafylline, and ketoconazole being CYP2D6-, CYP2C9-, CYP1A2-, and CYP3A4-selective inhibitors, respectively (9, 12)].

This assay methodology is efficient, easily automated, and uses commercially available, relatively low-cost materials. IC_{50} values can be determined quickly and reproducibly. This method shows promise for rapid, high-throughput screening of drug candidates for the specificity and potency of inhibition of cytochrome P450 enzymes.

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Direct Transfection of Polymerase Chain Reaction-Generated DNA Fragments into Mammalian Cells Employing Ethidium Bromide Indicator and Ultrafiltration

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Recombinant DNAs, small RNAs, and antisense oligonucleotides have been widely introduced into eukaryotic cells to study different aspects of molecular biology and pharmacology (1). Transfection methods (2) such as calcium phosphate, DEAE-dextran, electroporation, modified viruses, liposomes, and dendrimers (3) were employed showing advantages and limitations. In this

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